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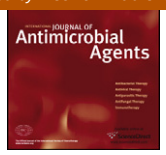

Efficacy of miltefosine treatment in *Leishmania amazonensis*-infected BALB/c mice

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ABSTRACT

Leishmaniasis is one of the most serious worldwide diseases caused by protozoan parasites of the *Leishmania* genus, affecting millions of people around the world. All currently available treatments present severe toxic side effects, require long-term compliance, cause serious side effects and are uncomfortable for patients. *Leishmania amazonensis*, a species endemic to Brazil, causes severe localised or diffuse skin lesions in humans. Owing to the unsatisfactory nature of the currently available chemotherapies, new approaches have been assessed for improved therapeutic intervention strategies against leishmaniasis. Miltefosine is an alkylphospholipid analogue that exhibits potent activity against the different clinical manifestations of leishmaniasis. Thus, the aim of this study was to investigate the long-term efficacy of miltefosine in BALB/c mice infected with *L. amazonensis* owing to the lack of a profound study demonstrating its dose-dependent and long-term effects. It was observed that animals treated with 20–50 mg/kg/day of miltefosine exhibited a significant dose-dependent reduction in lesion size; furthermore, in mice receiving higher doses, lesions disappeared after the end of treatment. To confirm a possible parasitological cure, mice up to 250 days after the end of treatment were analysed. No lesions or presence of parasite DNA were found in mice treated with 30, 40 and 50 mg/kg/day of miltefosine. In summary, these results show that miltefosine may be used to treat cutaneous leishmaniasis caused by *L. amazonensis*, alone or as combination therapy.

1. Introduction

Leishmaniasis is a complex of mammalian diseases caused by protozoan parasites of the *Leishmania* genus, infecting over 12 million people around the world [1]. A total of 21 *Leishmania* spp. have been described and together they are responsible for the three main clinical manifestations of leishmaniasis, namely cutaneous, mucocutaneous and visceral leishmaniasis. *Leishmania amazonensis* is an important species in Brazil that is capable of producing a wide spectrum of human diseases, including the diffuse (anergic) cutaneous leishmaniasis (CL) that is highly mutilating and incurable using currently available forms of treatment [2,3].

Although the treatment for leishmaniasis was introduced in the early 20th century, parenteral administration of pentavalent antimony compounds (meglumine antimoniate and sodium stibogluconate) remains the first-choice treatment for all forms of leishmaniasis [4]. In the case of antimonial resistance, the second-choice treatment includes pentamidine and amphotericin B (deoxycholate or liposomal formulation) [4]. However, each of these therapies presents important limitations, including long-term parenteral administration, toxic side effects, high cost in endemic countries, and a high number of resistance cases predominately associated with human immunodeficiency virus (HIV) co-infection [4]. Thus, these drugs are unsatisfactory and there is an urgent need to identify novel compounds or therapeutic strategies that are more safe, accessible and less toxic.

Miltefosine, a hexadecylphosphocholine, was initially studied as an anti-tumour agent [5]; more recently, it was described to exhibit both in vitro and in vivo activity against *Leishmania* parasites [6–11]. Oral administration of miltefosine has been used in India to treat visceral leishmaniasis (VL) since 1998.

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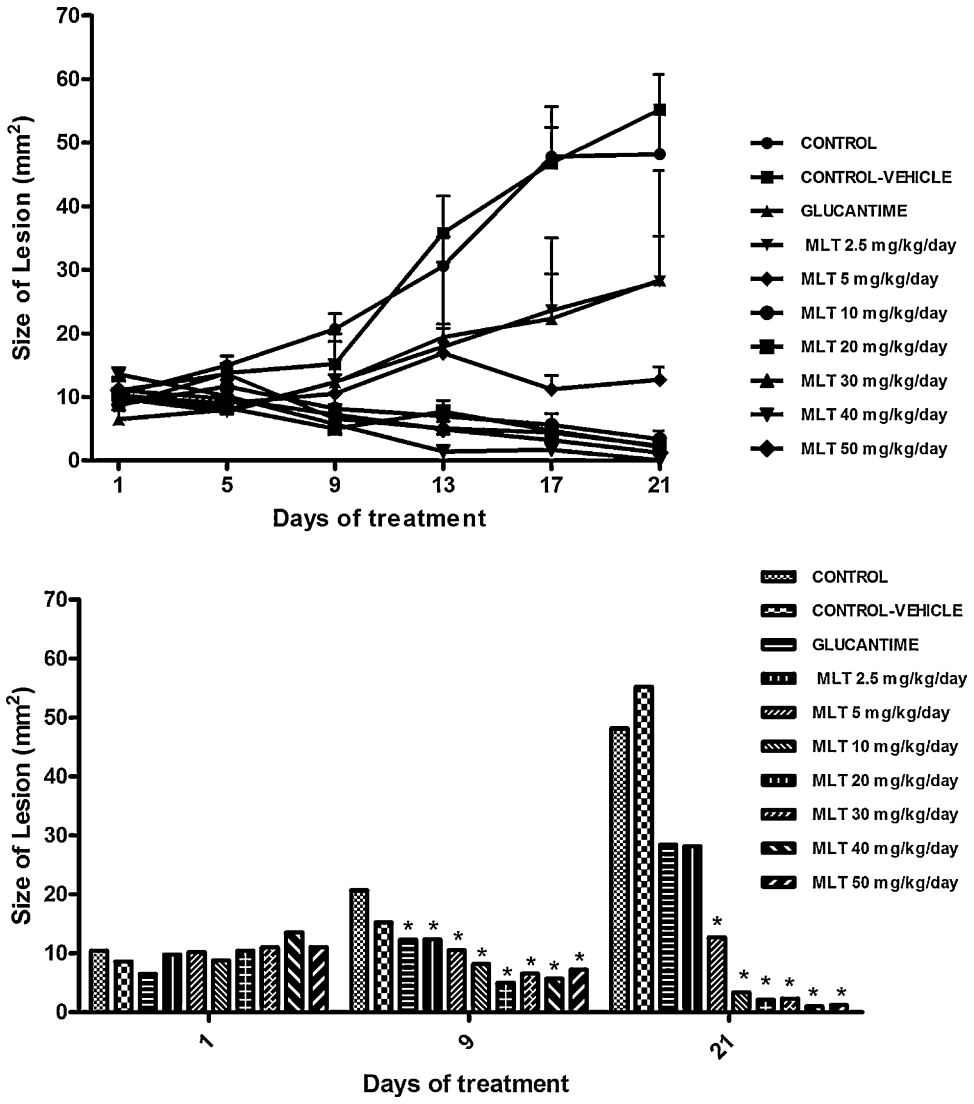

Fig. 1. In vivo efficacy of Glucantime® and oral miltefosine (MLT) treatment in BALB/c mice infected with *Leishmania amazonensis*. Infection and treatment are described in Sections 2.1 and 2.2. (A) Dose–response curve of MLT treatment. (B) Showing the significant effects of MLT after 9 days and 21 days of treatment. Lesion sizes were monitored weekly. Values are the mean of lesion sizes in five mice in each group and bars represent the standard deviation. $^{*}P < 0.05$ comparing all doses of Glucantime and MLT with the control group.

Currently, it is recommended in India and Ethiopia to treat VL and in Colombia, Bolivia and Guatemala to treat CL [12–19]. In Brazil, recent studies demonstrated that miltefosine is also efficient against infections caused by *Leishmania braziliensis* [20], *Leishmania guyanensis* [21] and *Leishmania chagasi* [22]. Miltefosine can be considered less severe than the other drugs, however it presents some important side effects such as gastrointestinal disorders and teratogenicity [23,24]; thus, it is not recommended for women of fertile age. Although several works have demonstrated the efficacy of miltefosine, it remains an interesting compound to investigate its activity against different *Leishmania* spp., particularly species associated with cutaneous infections in Brazil.

Thus, the present study investigated the efficacy of miltefosine treatment for experimental CL in *L. amazonensis*-infected BALB/c mice using seven doses varying from 2.5 mg/kg/day to 50 mg/kg/day and treatment lasting 21 days. To confirm the long-term efficacy of miltefosine, treated mice were evaluated 250 days after treatment ended by measurement of lesion size, skin smears and quantitative polymerase chain reaction (qPCR).

2. Materials and methods

This study (protocol no. IBCCF 096/097) was approved by the Ethics Committee for Animal Experimentation of the Health Sciences Centre, Federal University of Rio de Janeiro (Brazil). All animals received humane care in compliance with the '*Principles of laboratory animal care*' formulated by the National Society for Medical Research and the '*Guide for the care and use of laboratory animals*' prepared by the National Academy of Sciences (Washington, DC).

2.1. Parasites and infections

Amastigotes of *L. amazonensis* WHOM/BR/75/Josefa strain were isolated from footpad nodules of infected BALB/c mice and were transformed into promastigotes in Warren's medium containing 10% foetal bovine serum at 25 °C. Following transformation, stationary promastigotes (5 days of culture in Warren's medium) were used to inoculate 5-week-old female BALB/c mice weighing ca. 20 g. A total of 60 mice were trichotomised at the base of the tail and were

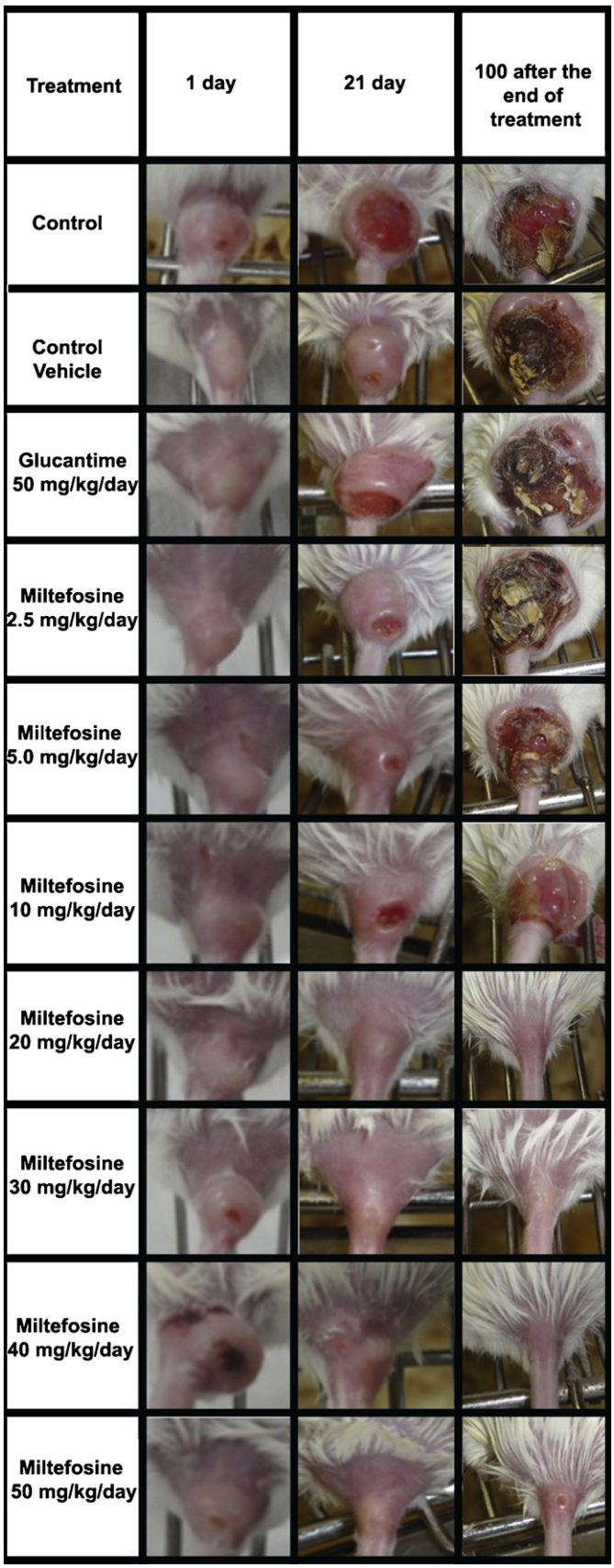

Fig. 2. Images of lesions during treatment of BALB/c mice infected with *Leishmania amazonensis*. Photographs were taken on the first and last days of treatment and 100 days after the end of treatment. In the control, vehicle control and Glucantime® groups, the lesions showed an intense swelling and ulcerated by 100 days after treatment cessation. In groups treated with miltefosine at doses ≥20 mg/kg/day, the photographs revealed a complete healing of the nodules and ulcers.

then inoculated with ca. 1.0×10^7 infective promastigotes/mouse by subcutaneous injection.

2.2. Treatment of infected mice

Treatment was initiated when the lesions reached a diameter of 5–7 mm (ca. 10 mm^2 lesion area), which in this experiment was exactly 4 weeks post infection. This initial condition of lesion size is important to mimic the real-life scenario when patients seek treatment services. A dose–response study with miltefosine (Cayman Chemical Co., Ann Arbor, MI) was carried out compared with meglumine antimoniate (Glucantime®; Aventis, São Paulo, Brazil) (a gift from the Health Secretary of Rio de Janeiro, Brazil) as a standard treatment. Miltefosine was administered by oral gavage, whilst Glucantime was given by intraperitoneal injection. Treatments were administered daily for 21 days. Stock solutions of miltefosine were prepared in polyethylene glycol 200 (PEG200) (Sigma, St Louis, MO), whilst Glucantime was diluted in water; both solutions were stored at 4 °C.

Following lesion development, mice were selected according to lesion size to ensure similar levels of infection and were then divided into ten groups (five mice per group). Treatment groups were as follows: control group treated with water; vehicle control group treated with PEG200; Glucantime group treated with 50 mg/kg/day Glucantime; and seven miltefosine groups treated with seven different doses of miltefosine (2.5, 5, 10, 20, 30, 40 and 50 mg/kg/day).

2.3. Treatment evaluation

Treatment efficacy was evaluated using three different parameters: (i) lesion size; (ii) lesion parasite burden as determined by Giemsa-stained skin smears; and (iii) tissue parasite burden as determined by real-time qPCR. During treatment, lesion size was monitored weekly using a calliper (Mitutoyo, Taipei, Taiwan) to determine the lesion diameter. In addition, weekly pictures of the lesions were taken. Lesion size was determined by obtaining the average value between horizontal and vertical directions, and the ulcer area was expressed in mm^2. Skin smears were made on glass slides, fixed with absolute methanol, stained with Giemsa and observed using a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) to check for the presence of amastigotes or infected macrophages.

To confirm the long-term efficacy and a possible parasitological cure of the miltefosine-treated mice, the different groups were followed for 250 days after the end of treatment. Mice were considered cured if lesions and ulcers were completely absent 100 days after the end of treatment.

2.4. Quantification of tissue parasite burden by quantitative polymerase chain reaction

Quantification of tissue parasite load was done by qPCR using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. DNA was isolated from lesions of infected mice at the end of the 21-day treatment period and also at 100 days and 250 days post treatment as described previously [25]. Briefly, samples were digested with 200 μg/mL proteinase K in 10 mM Tris–HCl (pH 7.6), 0.1 M NaCl, 10 mM ethylene diamine tetra-acetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS) for 2 h at 55 °C. Next, phenol–chloroform extraction and ethanol precipitation were performed according to standard protocols. For qPCR assays, samples were run in triplicate, where 1 ng of total DNA was added to a 15 μL reaction mixture containing Power SYBR Master Mix (Applied Biosystems). qPCR

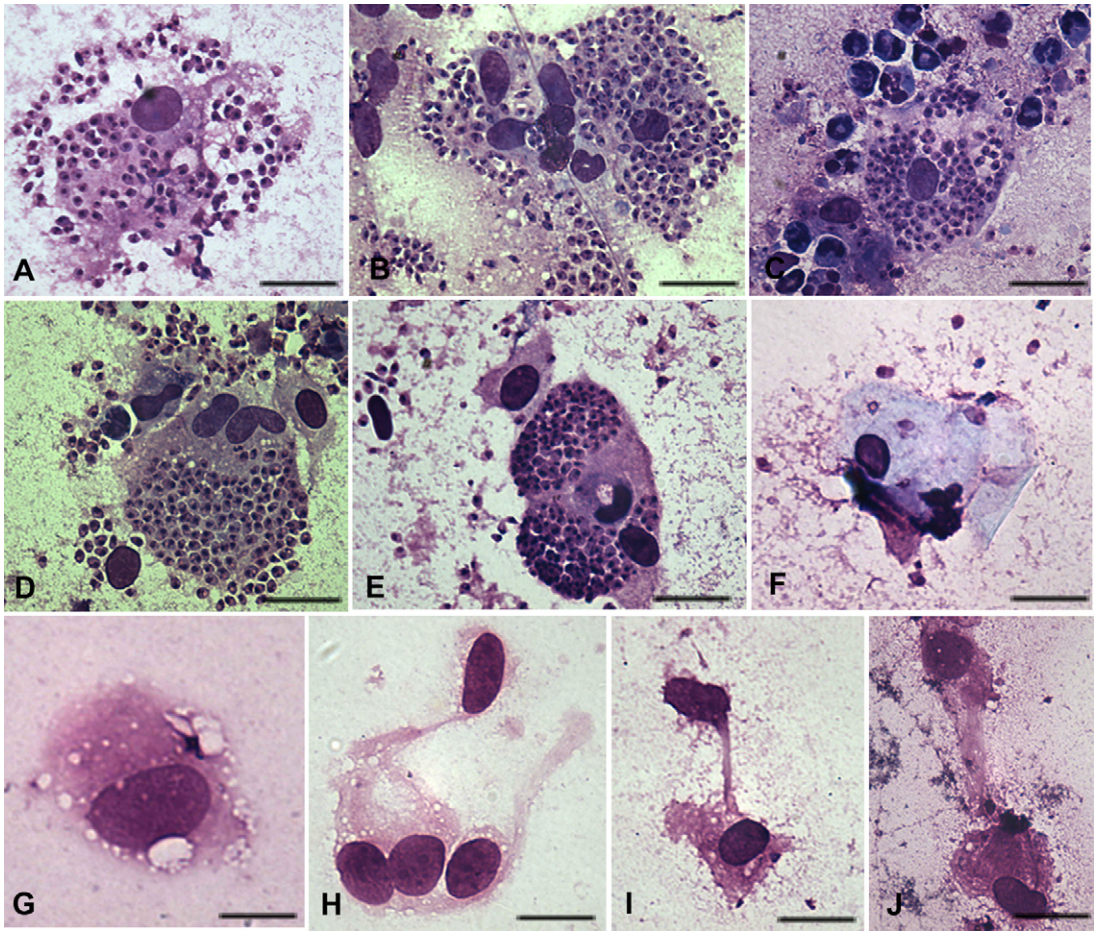

Fig. 3. Parasite burden throughout the course of treatment. Skin smears were taken from all experimental groups, stained with Giemsa and observed under a light microscope: (A) control; (B) vehicle control; (C) Glucantime 50 mg/kg/day; (D) miltefosine 2.5 mg/kg/day; (E) miltefosine 5 mg/kg/day; (F) miltefosine 10 mg/kg/day; (G) miltefosine 20 mg/kg/day; (H) miltefosine 30 mg/kg/day; (I) miltefosine 40 mg/kg/day; and (J) miltefosine 50 mg/kg/day. In the control and vehicle control groups and those treated with Glucantime® or miltefosine at doses of 2.5 mg/kg/day and 5 mg/kg/day groups, the skin smears appeared densely parasitised (A–E). In the group that received miltefosine at a dose of 10 mg/kg/day, few parasites were observed in the many fields that were searched (F). No parasites were seen in the groups treated with miltefosine at doses ≥20 mg/kg/day (G–J). Bars, 50 μm.

conditions were as follows: 55 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 58 °C for 60 s. *Leishmania* 18S RNA (5′-ACCGCCCGTCGTTGTTT-3′ and 5′-CACCGCCTGTCCGATCAC-3′) and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-TTCTGATCTCAGCTCCCCTGTT-3′ and 5′-CCAGGCGCCCAATACG-3′) primers were used. Purified DNA from *L. amazonensis* and mice was used for calibrating standard curves. The efficiency and specificity of the PCR reaction were evaluated using serial dilutions of each template DNA and melt curve analysis. The amount of DNA in the samples was determined using 7500 Sequence Detection Software (Applied Biosystems) by interpolating the threshold cycle (Ct) of each sample in the corresponding standard curve.

2.5. Statistical analysis

The statistical significance of differences in the average lesion diameters amongst groups was evaluated using the one-way analysis of variance (ANOVA) test followed by Tukey's test (GraphPad Prism 5 software; GraphPad Software Inc., La Jolla, CA). Differences were considered significant when *P*-values were <0.05.

3. Results and discussion

Treatment started at 4 weeks post infection when lesions were established and apparent. All mice in the treatment groups exhibited similar-sized lesions. Over the course of treatment, lesions grew normally according to the course of infection in the control and vehicle groups that did not receive treatment. In Glucantime-treated mice, lesion size decreased during treatment. In the groups that received oral miltefosine, the response was dose-dependent, with a reduction in lesion size during the 21-day treatment period (Fig. 1). For doses >5 mg/kg/day, the reduction in lesion size was statistically significant at 21 days after the end of treatment (Fig. 1B). Mice treated with 2.5 mg/kg/day of miltefosine, which is the dose recommended by the World Health Organization (WHO) and used to treat human VL, were similar to mice treated with 50 mg/kg/day Glucantime (Fig. 1). Photographs of all groups on the first and last days of treatment revealed the potent effect of miltefosine against *L. amazonensis*-infected mice (Fig. 2). In mice that did not receive treatment (control and vehicle control), lesions swelled at the infection site (Fig. 2). In the groups treated with Glucantime and with miltefosine at doses of 2.5, 5 and 10 mg/kg/day, lesions exhibited a smaller amount of swelling at the infection site

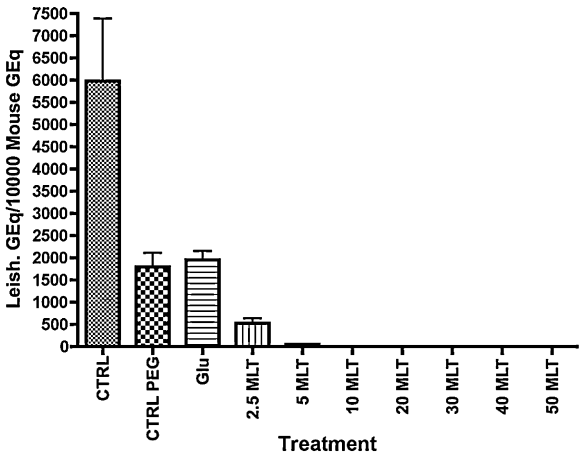

Fig. 4. Quantitative polymerase chain reaction (qPCR) of *Leishmania amazonensis* in skin lesions of infected mice. Assays were performed as described in Section 2.4 and results are expressed as *Leishmania* genome equivalents (GEq) per 10 000 mouse GEq considering the amount of DNA per cell (0.12 pg in *Leishmania* [29] and 12 pg in mouse [30]). Error bars indicate the standard error. CTRL, control group; CTRL PEG, treated with polyethylene glycol 200; Glu, treated with Glucantime®; 2.5–50 MLT, treated with 2.5–50 mg/kg/day of miltefosine.

compared with the control group at the 21-day treatment period (Fig. 2). Mice treated with miltefosine at doses of 20, 30, 40 and 50 mg/kg/day did not exhibit nodules or ulcers (Fig. 2).

Parasite burden in Glucantime- and miltefosine-treated animals was evaluated by microscopic analysis of skin smears stained with Giemsa. At the 21-day treatment period, mice in the control and vehicle control groups (that received water and PEG200), respectively, the Glucantime group and the miltefosine groups that received doses of 2.5 mg/kg/day or 5 mg/kg/day presented a large number of amastigotes inside macrophages at the lesion site (Fig. 3A–E). In mice treated with 10 mg/kg/day of miltefosine, the number of amastigotes was significantly reduced in comparison with the control groups and the groups treated with Glucantime and miltefosine at doses of 2.5 mg/kg/day and 5 mg/kg/day (Fig. 3F). Interestingly, amastigotes were not observed in the skin smears from mice treated with doses ≥20 mg/kg/day of miltefosine (Fig. 3G–J).

To confirm the efficacy of miltefosine at the 21-day treatment period, parasite burden was also evaluated by detecting parasite DNA in lesions by qPCR analysis. As depicted in Fig. 4, parasite burden in Glucantime- and PEG200-treated animals was reduced ca. three-fold compared with the control group. Treatment with 2.5 mg/kg/day of miltefosine reduced parasite burden ca. 10-fold, whilst miltefosine doses ≥5 mg/kg/day led to parasite DNA levels at or below the limit of detection. These results were consistent with the clinical lesion progression shown in Figs. 2 and 3.

During the 100 days after the end of treatment, lesions in animals that did not receive treatment and those treated with Glucantime or miltefosine at doses varying from 2.5 mg/kg/day to 10 mg/kg/day were swelled at the infection site and became ulcerated (Fig. 2), whilst mice treated with miltefosine at doses of 20, 30, 40 and 50 mg/kg/day did not exhibit nodules or ulcers (Fig. 2). However, skin smears revealed the presence of intracellular amastigotes in mice treated with doses of 20 mg/kg/day of miltefosine but not in mice treated with doses of 30, 40 and 50 mg/kg/day of miltefosine (data not shown).

To confirm a possible parasitological cure, we decided to investigate treated mice during 250 days after the end of treatment, different from other studies that have evaluated the short-term efficacy of miltefosine against *L. amazonensis*-infected mice [26]. During this time, lesion appearance was observed. Skin smears and qPCR were also made and analysed to determine parasite burden. Fig. 5 depicts lesion development 250 days after treatment was terminated. Complete clinical cure was observed in mice that received miltefosine at doses of 30, 40 and 50 mg/kg/day; these mice did not present any nodules or ulcers and were healthy in appearance. In addition, qPCR analysis confirmed the clinical cure, where parasite DNA was not detected in the tissues collected, including liver and spleen (data not shown).

Previous studies demonstrated the efficacy of miltefosine against murine models of VL caused by *Leishmania donovani* [10] and CL caused by *Leishmania major* [27], *Leishmania mexicana* [28] and *L. amazonensis* [26]; however, these studies evaluated the short-term efficacy of miltefosine. In animal models, the efficacy doses are >10 mg/kg/day. Sometimes doses of 20 mg/kg/day or combination therapy are required to have an effective reduction of the parasite burden in the liver, spleen and skin [14,30]. Although mice treated with 50 mg/kg/day of miltefosine were cured, they presented signs of toxicity, with significant weight loss; however, no animals died during the 21 days of treatment.

In summary, these results showed that at doses ≥30 mg/kg/day, miltefosine is efficacious in the treatment of *L. amazonensis*-infected mice. Effective cure was observed at miltefosine doses of 30, 40 and 50 mg/kg/day after 250 days of observation, which was confirmed by qPCR analysis. Thus, miltefosine remains a promising compound to use against the different clinical manifestations of leishmaniasis. Furthermore, as its side effects could be considered minimal compared with the effects observed for Glucantime,

Fig. 5. Lesion appearance 250 days following treatment cessation. Photographs of infected mice treated with (A) 30 mg/kg/day, (B) 40 mg/kg/day and (C) 50 mg/kg/day of miltefosine were taken 250 days after treatment was stopped. These photos demonstrate a clinical healing of the infection, where no nodules or ulcers were seen at the base of the tail. At this time, the treated mice appear completely healthy.

pentamidine and amphotericin B, it could be utilised alone or in combined therapy to reduce the therapeutic dose. Therefore, this study confirmed the long-term efficacy of miltefosine to treat CL caused by Brazilian species of *Leishmania*, thus supporting the need for the advancement of miltefosine clinical trials.

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Competing interests: None declared.

Ethical approval: This study (protocol no. IBCCF 096/097) was approved by the Ethics Committee for Animal Experimentation of the Health Sciences Centre, Federal University of Rio de Janeiro (Brazil). All animals received humane care in compliance with the '*Principles of laboratory animal care*' formulated by the National Society for Medical Research and the '*Guide for the care and use of laboratory animals*' prepared by the National Academy of Sciences (Washington, DC).

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